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Aggregation of Human Platelets by Acidic Mucopolysaccharide Extracted from *Stichopus japonicus* Selenka

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Key words

Platelet aggregation – Acidic mucopolysaccharide – Humans

Summary

The acidic mucopolysaccharide extracted from sea cucumber (*Stichopus japonicus* Selenka) (SJAMP) has been shown to cause platelets to aggregate. Using citrated platelet-rich plasma (PRP), washed platelets and formaldehyde-fixed platelets from humans, we investigated the effects of platelet inhibitors and various plasmas and their fractions on SJAMP-induced platelet aggregation. It was found that the lowest concentration of SJAMP required for the aggregation of human platelets was about 0.4 μg/ml and the magnitude of aggregation induced by SJAMP was concentration dependent. The platelets were aggregated by SJAMP at 10 μg/ml in 25 out of 28 (89%) normal subjects tested. Platelet inhibitors such as PGE_1, aspirin, indomethacin, apyrase, antimycin, 2-deoxy-D-glucose and EDTA inhibited by 70 to 100% the aggregation induced by SJAMP. Washed platelets alone were not aggregated by SJAMP. In the presence of fibrinogen, washed platelets were aggregated by SJAMP, but formaldehyde-fixed platelets were not. These data indicate that the SJAMP-induced human platelet aggregation requires extracellular calcium, fibrinogen, and energy metabolism. The second phase of aggregation is dependent upon the release of ADP, and cyclooxygenase pathway.

Introduction

The acidic mucopolysaccharide extracted from sea cucumber (*Stichopus japonicus* Selenka) (SJAMP) is a heparin-like substance with a molecular weight ranging from 30,000 to 50,000, containing galactosamine, glucuronic acid, fucose and sulfate (1). SJAMP possesses an antithrombin-like activity even in the absence of antithrombin III or other plasma factors (2, 3). Its antithrombin-like activity in the whole plasma is weaker than that of heparin (2, 3). After intramuscular injection into humans, SJAMP causes prolongation of thrombin time (4). It has also been demonstrated that SJAMP causes the clumping of platelets from humans, rabbits, rats and mice (2, 3). In this paper, we report the effects of platelet inhibitors, various plasmas and their fractions on the aggregation of human platelets by SJAMP.

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Materials and Methods

Materials

Aspirin, indomethacin, apyrase (Grade III), ethylene diaminetetraacetic acid (EDTA), 2-deoxy-D-glucose, antimycin, PGE_1, hirudin, adenosine diphosphate, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO. N-ethylmaleimide was purchased from Mallinkrodt Chemical, St. Louis, MO. Heparin was manufactured by Lyphomed, Inc., Chicago, IL. D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone 2 HCl (PPACK) was produced by Boehringer Mannheim Biochemicals, Indianapolis, IN. Fibrinogen was supplied by A. B. Kabi, Stockholm, Sweden. SJAMP was generously provided by Dr. H.-Z. Fan, Tianjin Medical and Materia Medica Institute, Tianjin, China. Other chemicals were of analytical grade.

Preparation of Reagents and Buffers

PGE_1, indomethacin and antimycin were dissolved in pure ethanol, aliquoted and stored at −20° C. Before use, they were diluted with Tris-HCl saline buffer, pH 7.4, containing 0.05 M Tris-HCl and 0.15 M NaCl. Aspirin, apyrase, EDTA, 2-deoxy-D-glucose, and N-ethylmaleimide were freshly prepared. PPACK was dissolved in 1 mM HCl and diluted with Tris-HCl saline buffer pH 7.4 prior to use.

Calcium-free Tyrode's buffer pH 6.8 or 7.4 was prepared immediately before use containing 8.0 g/l NaCl, 0.2 g/l KCl, 0.05 g/l NaH_2PO_4, 0.415 g/l $MgCl_2 \cdot 6H_2O$, 1.0 g/l $NaHCO_3$ and 1.0 g/l dextrose; the pH was adjusted to 6.8 or 7.4 and the osmolality was adjusted to 290–300 mOsm with water or 1 M NaCl.

Preparation of Platelet-Rich Plasma, Washed Platelet Suspension, and Formaldehyde-Fixed Platelets

Nine parts of blood were drawn from the antecubital vein into one part of 3.8% sodium citrate by double syringe technique from 28 normal subjects (20 males and 8 females) with age ranging from 20 to 53 years. All of them had not taken any antiplatelet drug for 14 days prior to drawing of blood. After centrifugation at 180 g for 10 min at 22° C, the supernatant (platelet-rich plasma, PRP) was aspirated and kept at 22° C until use.

Platelets were washed according to the method of Walsh (5) with some modification. The platelet-rich plasma was transferred to a conical plastic tube. A volume of 35% bovine serum albumin equal to 1/25 volume of PRP was introduced to the bottom as cushion. After centrifugation at 1600 g for 15 min at 22° C, the supernatant was discarded. The platelets were suspended in calcium free Tyrode's buffer, pH 6.8 and 1 unit/ml of apyrase. The washing was repeated twice. Finally, platelets were suspended in Tyrode's buffer, pH 7.4 with 0.5 mM Ca^{2+} and adjusted to 750 × 10^9/l. The formaldehyde fixed platelets were prepared according to the method of Macfarlane et al. (6). After the PRP was incubated at 37° C for 1 h, it was mixed with an equal volume of 2% formaldehyde and then kept at 4° C overnight (at least 18 h). After centrifugation at 2000 g for 15 min at 4° C, the platelet pellet was washed with cold Tris-HCl saline buffer, pH 7.4, containing 0.05 M Tris-HCl and 0.15 M NaCl at 4° C three times. Then platelets were suspended in Tris-HCl saline buffer, pH 7.4 and adjusted to 750 × 10^9/l. The formaldehyde fixed platelets were kept at 4° C for less than 1 week.

Preparation of Platelet-Poor Plasmas and Sera

The human platelet-poor plasma was prepared by utilizing the residual blood after the platelet-rich plasma had been collected. The residual blood was centrifuged at 2400 g for 20 min at 4° C. The rabbit (New Zealand White) blood was drawn from the central aural artery by a butterfly infusion set. The first 3 to 4 drops were discarded, and thereafter blood was collected into a plastic tube containing 3.8% sodium citrate solution. The ratio of blood to 3.8% sodium citrate was 9:1. The platelet-poor plasma was collected from supernatant after centrifugation of citrated blood at 2400 g for 20 min at 4° C. The sera were harvested after incubation of whole blood at 37° C for 4 h.

Fractionation of Plasma with Ammonium Sulfate

Citrated plasma was mixed with 1/30 volume of aluminum hydroxide (Amphojel, Wyeth Laboratories, Inc., Philadelphia, PA) dropwise with constant stirring. The mixture was incubated with stirring at 37° C for 30 min. After centrifugation at 10,000 g for 30 min, the adsorbed supernatant plasma was brought to 20% ammonium sulfate saturation by the addition of saturated ammonium sulfate solution, pH 7.4. The suspension was stirred at 4° C for 30 min and the precipitate was removed by centrifugation at 10,000 g for 30 min. The supernatant was again mixed with saturated ammonium sulfate solution and brought to a certain percent saturation; the precipitate was obtained after centrifugation as described. The procedures were repeated several times to obtain precipitates at 0–20%, 20–30%, 30–40%, 40–50% and 50–60% saturated ammonium sulfate fractions. Each precipitate was dissolved in 1/10 volume (referred to the original volume of plasma) of Tyrode's buffer, pH 7.4. After dialysis against the 100 volume of same buffer with 3 changes for 24 h at 4° C, the solutions were stored at −70° C.

Platelet Aggregation Studies

Platelet aggregation was carried out according to the method described by Born (7) using a Chrono-log platelet aggregometer (Chrono-log Corporation, Havertown, PA). 0.4 ml of PRP (containing approximately 200 to 300 × 10^3/μl platelets) was incubated in a cuvette at 1,200 rpm at 37° C for 3 min and then 2 μl of platelet aggregating agent was added. The change of optical density as a result of platelet aggregation was recorded.

Study of the Effects of Serum, Plasma and Its Fractions on the Aggregation of Washed Platelets

A mixture of 0.15 ml of washed platelet suspension containing 750 × 10^9/l platelets, 0.15 ml of Tyrode's buffer, pH 7.4 and 0.1 ml of serum, plasma, plasma fraction or human fibrinogen solution was incubated in a cuvette at 37° C for 3 min and subsequently, 2 μl of solution containing 4 μg of SJAMP was added. Change of optical density as a result of platelet aggregation was recorded.

Study of the Effects of Platelet Inhibitors and Thrombin Inhibitors on the SJAMP-Induced Platelet Aggregation

A mixture of 0.35 ml of PRP and 0.05 ml of inhibitor or Tyrode's buffer was incubated at 37° C for 5 min in a cuvette in the Chrono-log aggregometer. Subsequently, 2 μl of solution containing 4 μg of SJAMP was added. The percent decrease of optical density resulting from platelet aggregation with or without inhibitor was recorded and compared.

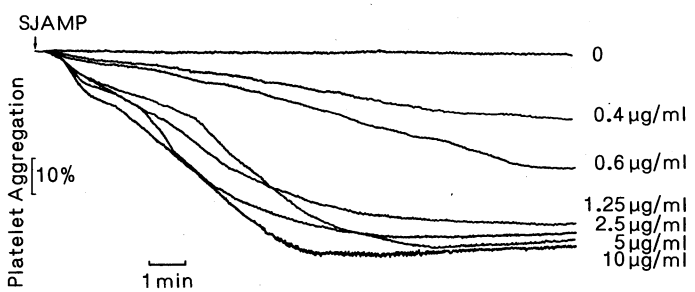

Fig. 1 The magnitude of aggregation of human platelets in PRP from a normal subject who had more than 50% maximal aggregation induced by 10 μg/ml of SJAMP is dependent on the concentration of SJAMP

Table 1 The magnitude of maximal aggregation of platelets in the platelet-rich plasma from 28 subjects induced by SJAMP (10 μg/ml) and ADP (2.5 μM)

Magnitude of maximal aggregation	Number of subjects SJAMP	ADP
	28 (100%)	28 (100%)
0–10%	3 (11%)	0 (0)
11–30%	5 (18%)	1 (4%)
31–50%	8 (28%)	1 (4%)
>50%	12 (43%)	26 (92%)

Study of the Effects of SJAMP on Formaldehyde-Fixed Platelets

A mixture of 0.15 ml formaldehyde-fixed human platelet suspension (750 × 10^9/l), 0.1 ml of human platelet-poor plasma, and 0.15 ml of Tyrode's buffer, pH 7.4 was incubated in a cuvette in the Chrono-log aggregometer at 37° C for 5 min. Following this, 2 μl of solution containing 4 μg of SJAMP was added. The percent decrease of optical density resulting from platelet aggregation was recorded.

Results

Magnitude of the Aggregation of Human Platelets Induced by SJAMP

At the final concentration of 10 μg/ml SJAMP, among the 28 normal subjects, there was almost no aggregation of platelets in 3, 11–30% in 5, 31–50% in 8, and >50% in 12; therefore, 25 out 28 (89%) normal subjects had platelet aggregation induced by SJAMP at 10 μg/ml. In comparison, the magnitude of aggregation by ADP on the same samples at the concentration of 2.5 μM was 11–30% in 1, 31–50% in 1 and >50% in 26 (Table 1).

In the aggregation of human platelets induced by SJAMP, there was a delay of about 20 to 40 s before aggregation started. The aggregation was irreversible. About 71% of aggregation by SJAMP at 10 μg/ml was shown to have two phases. The magnitude of aggregation was dependent on the concentration of SJAMP (Fig. 1). When the concentration of SJAMP was higher than 60 μg/ml, there was a prolonged delay period and a slight increase of optical density initially (Fig. 2). The reaction mixture in the cuvettes was examined by phase microscopy. The platelet aggregates were present and their size was proportional to the concentrations of SJAMP added.

Within 2 h after drawing of blood, the magnitude of platelet aggregation induced by SJAMP showed no significant change. When greater than 3 h of time had elapsed at 22° C, the magnitude of platelet aggregation induced by SJAMP decreased dramatically, to 10% of initial maximal aggregation by 6 h. In contrast, ADP-induced aggregation did not change as much over time so that all the experiments using PRP were done within 2 h after drawing of blood. When washed platelets were stored at 22° C for 5 h and then mixed with plasma and Tyrode's buffer, pH 7.4, there was no significant change in platelet aggregation.

Effects of Sera, Plasmas, Plasma Fractions, and Human Fibrinogen on the Aggregation of Washed Platelets by SJAMP

Washed human platelets alone were not aggregated by SJAMP. The platelet aggregation was restored upon addition of human or rabbit plasma, but not human or rabbit serum (Fig. 3).

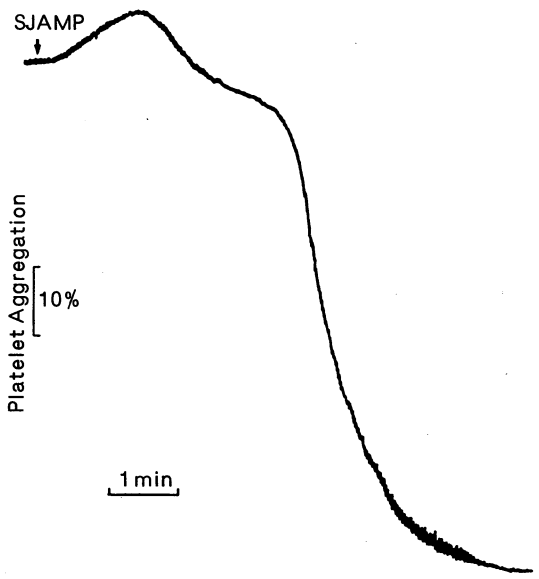

Fig. 2 Human platelet aggregation induced by high concentration of SJAMP (60 μg/ml)

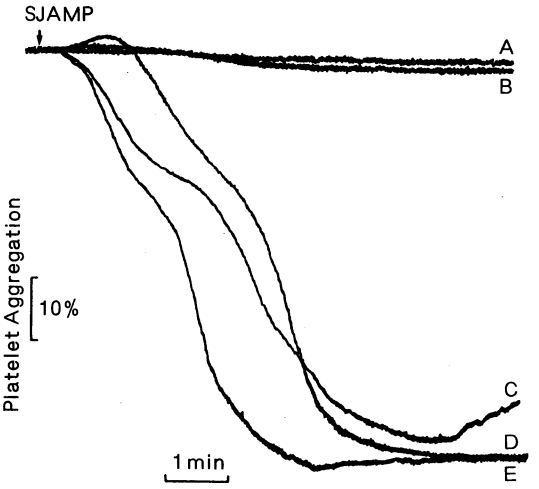

Fig. 3 The effect of plasmas on the aggregation of washed human platelets by SJAMP (10 μg/ml). To a mixture of 0.15 ml washed platelet suspension (750 × 10^9/l) and 0.15 ml of Tyrode's buffer, 0.1 ml of human serum (A), rabbit serum (B), rabbit plasma (D) or human plasma (E) was admixed before addition of SJAMP. The PRP (C) was used for comparison

When plasma fractions separated by ammonium sulfate saturation were used, only the 0 to 30% ammonium sulfate saturated fraction of human or rabbit plasma restored the platelet aggregation induced by SJAMP. Since the human and rabbit sera failed to recover the platelet aggregating activity, it was suspected that fibrinogen is required for the aggregation. Upon addition of human fibrinogen, the platelet aggregatory activity was restored (Fig. 4). The minimal concentration of human plasma or fibrinogen required in the platelet aggregation mixture was approximately 12% of human plasma or 240 μg/ml of human fibrinogen, respectively.

Effects of Platelet Inhibitors and Thrombin Inhibitors on SJAMP-Induced Platelet Aggregation

As shown in Table 2, aspirin (1 mM) and indomethacin (8 mM), inhibitors of cyclooxygenase, inhibited the SJAMP-induced platelet aggregation. Apyrase (15 units/ml), which removes ADP, suppressed 80% of aggregation induced by SJAMP. Antimycin (1.4 μM) and 2-deoxy-D-glucose (8 mM), energy metabolic inhibitors, inhibited 84 and 90% of aggregation induced by SJAMP respectively. When both agents were used together, the aggregation was inhibited completely. PGE_1 (1 μM), which increases platelet cAMP, completely inhibited the aggregation by SJAMP. N-ethylmaleimide (50 mM), a sulfhydryl-group blocking agent, reduced 85% of aggregation by SJAMP. EDTA at 4 mM, a Ca^{2+} chelating agent, completely abolished the

Table 2 Effect of inhibitors on SJAMP-mediated platelet aggregation

Inhibitor	Concentration	Aggregation* (%)	Inhibition* (%)
Control		66	
Aspirin	1 mM	18	73
Indomethacin	8 μM	7	90
Apyrase	15 units/ml	14	79
Antimycin	1.4 μM	11	84
2-deoxy-D-glucose (DOG)	8 mM	7	90
Antimycin (1.4 μm) + DOG (8 mM)		0	100
PGE_1	1 μM	0	100
N-ethylmaleimide	50 μM	8	88
EDTA	4 mM	0	100
PPACK	1 μM	69	0
Heparin	10 units/ml	68	0
Hirudin	2.5 units/ml	70	0

* The data are representative from one of three separate experiments.

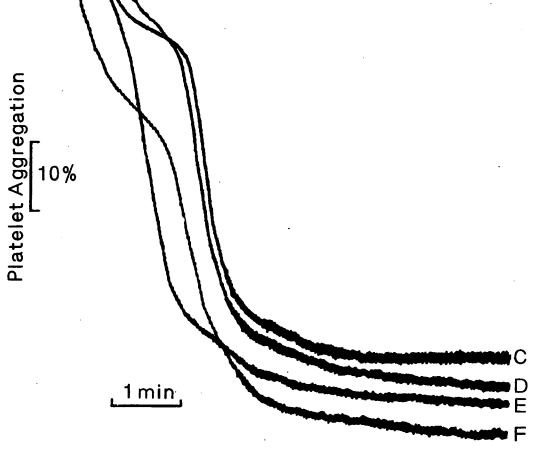

Fig. 4 The effect of various species sera, ammonium sulfate saturated fractions of plasma and human fibrinogen the aggregation of washed human platelets by SJAMP (10 μg/ml). To the mixture of 0.15 ml of washed platelet suspension (750 × 10^9/l) and 0.15 ml of Tyrode's buffer, 0.1 ml of human serum (A), rabbit serum (B), 0–30% ammonium sulfate saturated human plasma fraction (C), human fibrinogen (15 mg/ml) (D), or 0–30% ammonium sulfate saturated rabbit plasma fraction (E) was admixed before addition of 4 μg SJAMP. The PRP (F) was used for comparison

aggregation of SJAMP. The thrombin inhibitors (1 μm of PPACK, 10 units/ml of heparin and 2.5 units/ml of hirudin) did not have any effect on the SJAMP-induced aggregation.

Effect of SJAMP of Formaldehyde-Fixed Platelets

The formaldehyde fixed platelets suspended in plasma were not aggregated by SJAMP even at the high concentration of 30 μg/ml.

Discussion

The acidic mucopolysaccharide extracted from *Stichopus japonicus* Selenka caused the variable magnitude of aggregation of human platelets in 89% of normal subjects. The percentage of normal subjects who had SJAMP-induced platelet aggregation was higher than that reported by Brace and Fareed (8) in heparin-induced platelet aggregation. The aggregation of platelets by SJAMP decreased dramatically when platelet-rich plasma was stored more than 2 h at 22° C. However, when washed platelets were stored at 22° C for 5 h and then mixed with plasma in Tyrode's buffer, pH 7.4, there was no significant change in platelet aggregation. This suggests that the decrease of SJAMP-induced platelet aggregation, over time after blood drawing, is possibly caused by the decrease of pH due to evaporation of CO_2 in addition to decreased metabolism. This phenomenon is similar to what we observed in the platelet aggregation induced by heparin extracted from bovine lung or porcine intestinal mucosa (unpublished data).

The platelet aggregation induced by SJAMP revealed a delay period for about 20 s followed by two phase aggregation. This is similar to the aggregation induced by high concentration of heparin (approximately 70 units/ml, or 700 μg/ml) (14) and that caused by ADP. The minimal concentration of SJAMP required to induce platelet aggregation was 0.4 μg/ml, which was much lower than that of heparin. At 10 μg/ml, SJAMP induced two phase aggregation in 71% of subjects. In contrast, Salzman et al. reported that heparin from porcine intestinal mucosa at the 10 μg/ml concentration induced two phase aggregation in 5% of experiments (11).

The platelet aggregation induced by SJAMP was inhibited by EDTA (Ca^{2+} chelating agent), and by PGE_1 (a substance that elevates platelet cyclic AMP). This is similar to that induced by heparin (11). The SJAMP-induced platelet aggregation was partially inhibited by inhibitors of cyclooxygenase (such as aspirin and indomethacin) and by the removal of ADP using apyrase. This phenomenon is also observed in the aggregation induced by heparin (4, 12, 13, 16, 18). The SJAMP-induced platelet aggregation was shown to have two phases and the second phase was inhibited by aspirin and apyrase. These results indicate that the effect of SJAMP on human platelets is similar to that of other weak agonists (19).

We have demonstrated that the washed platelets were not aggregated by SJAMP, which suggests that a plasma cofactor is required. Upon addition of human plasma, or 0–30% ammonium sulfate saturated fraction of human or rabbit plasma which is rich in fibrinogen (20), the platelet aggregation was restored. Human and rabbit sera did not have any effect, suggesting that the plasma cofactor is fibrinogen. This speculation was confirmed by the observation that the aggregation of washed platelets was restored by addition of human fibrinogen. Salzman et al. (11) observed that platelet aggregation induced by heparin required a plasma cofactor different from that required for ristocetin-induced aggregation. The nature of plasma factor required for heparin-induced aggregation was not further investigated but it was pointed out that at least 75% of plasma present in the reaction mixture was required for the aggregation (11). In contrast, only 12% of plasma in the reaction mixture was needed for SJAMP-induced aggregation.

As heparin (11), but in contrast to ristocetin (6), SJAMP did not cause the agglutination of formaldehyde-fixed platelets. Both 2-deoxy-D-glucose (an inhibitor of glycolysis) and antimycin (an inhibitor of oxidative phosphorylation) inhibited the platelet aggregation induced by SJAMP. These suggest that SJAMP-induced platelet aggregation requires energy metabolism.

The effect of endotoxin, a lipopolysaccharide, on human platelets has been studied and remains controversial. Ream et al. (21) found that endotoxin caused the aggregation of human platelets in the presence of a heat-labile plasma factor (factor V). Other workers (22–24) reported that endotoxin had no effect on human platelets. Recently, Saba et al. (25) observed an inhibitory effect of endotoxin on human platelet aggregation induced by various aggregating reagents. In any event, the effect of SJAMP on human platelets is different from that of endotoxin and is not caused by contamination of endotoxin.

In conclusion, the SJAMP-induced aggregation requires energy metabolism, extracellular calcium ion, and a plasma cofactor, fibrinogen. The second phase of aggregation is dependent upon the release of ADP and cyclooxygenase pathway.

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